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<b>(21) International Application Number:</b> PCT/US96/01489 <b>(22) International Filing Date:</b> 1 February 1996 (01.02.96)  <b>(30) Priority Data:</b> 08/393,273 23 February 1995 (23.02.95) US  <b>(71) Applicant:</b> LUDWIG INSTITUTE FOR CANCER RE- SEARCH [CH/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).  <b>(72) Inventors:</b> BOON-FALLEUR, Thierry; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). VAN DER BRUGGEN, Pierre; Avenue Hippocrate 74, UCL 7459, B-1200 Brus- sels (BE). DE PLAEN, Etienne; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). LURQUIN, Christoph; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). GAUGLER, Beatrice; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). VAN DEN EYNDE, Benoit; Av- enue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). TRAVERSARI, Catia; Via Olgettina, 60, I-20132 Milano (IT). ROMERO, Pedro; 155, chemin des Boveresses, CH- 1066 Epalinges (CH).  <b>(74) Agent:</b> HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).		<b>(81) Designated States:</b> AU, CA, CN, FI, JP, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ISOLATED NONAPEPTIDES PRESENTED BY HLA MOLECULES, AND USES THEREOF		
<b>(57) Abstract</b>  The invention involves nonapeptides by HLA molecules such as HLA-A1. The resulting complexes are identified by cytolytic T cells. Such recognition may be used in diagnostics, or therapeutically.		

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**ISOLATED NONAPEPTIDES PRESENTED BY HLA  
MOLECULES, AND USES THEREOF**

This application is a continuation-in-part of copending application Serial No. 08/073,103 filed June 7, 1993, which is a continuation-in-part of copending application Serial Number 07/938,334, filed August 31, 1992, now U.S. Patent No. 5,405,940 and Serial Number 08/037,230 filed March 26, 1993.

**FIELD OF THE INVENTION**

This invention relates to immunogenetics and to peptide chemistry. More particularly, it relates to nonapeptides useful in various ways, including as immunogens and as binding partners for molecules such as HLA-A1. More particularly, it relates to so-called "tumor rejection antigens", presented by human leukocyte antigens of HLA-A1. These tumor rejection antigens provoke lysis of target cells to which they bind (via HLA molecules) by cytolytic T lymphocytes ("CTLs").

**BACKGROUND AND PRIOR ART**

The study of the recognition or lack of recognition of cancer cells by a host organism has proceeded in many different directions. Understanding of the field presumes some understanding of both basic immunology and oncology.

Early research on mouse tumors revealed that these displayed molecules which led to rejection of tumor cells when transplanted into syngeneic animals. These molecules are "recognized" by T-cells in the recipient animal, and provoke a cytolytic T-cell response with lysis of the transplanted cells. This evidence was first obtained with tumors induced in vitro by chemical carcinogens, such as methylcholanthrene. The antigens expressed by the tumors and which elicited the T-cell response were found to be different for each tumor. See Prehn, et al., J. Natl. Canc. Inst. 18: 769-778 (1957); Klein et al., Cancer Res. 20: 1561-1572 (1960); Gross, Cancer Res. 3: 326-333 (1943), Basombrio, Cancer Res. 30: 2458-2462 (1970) for general teachings on inducing tumors with chemical carcinogens and differences in cell surface antigens. This class of antigens has come to be known as "tumor specific transplantation antigens" or "TSTAs". Following the

5 observation of the presentation of such antigens when induced by chemical carcinogens, similar results were obtained when tumors were induced in vitro via ultraviolet radiation. See Kripke, J. Natl. Canc. Inst. 53: 333-1336 (1974).

10 While T-cell mediated immune responses were observed for the types of tumor described supra, spontaneous tumors were thought to be generally non-immunogenic. These were therefore believed not to present antigens which provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., Brit. J. Cancer 33: 241-259 (1976).

15 The family of tum<sup>-</sup> antigen presenting cell lines are immunogenic variants obtained by mutagenesis of mouse tumor cells or cell lines, as described by Boon et al., J. Exp. Med. 152: 1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum<sup>-</sup> antigens are obtained by  
20 mutating tumor cells which do not generate an immune response in syngeneic mice and will form tumors (i.e., "tum<sup>+</sup>" cells). When these tum<sup>+</sup> cells are mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum<sup>-</sup>"). See Boon et al., Proc. Natl. Acad. Sci. USA 74: 272 (1977), the  
25 disclosure of which is incorporated by reference. Many tumor types have been shown to exhibit this phenomenon. See, e.g., Frost et al., Cancer Res. 43: 125 (1983).

It appears that tum<sup>-</sup> variants fail to form progressive tumors because they initiate an immune rejection process. The  
30 evidence in favor of this hypothesis includes the ability of "tum<sup>-</sup>" variants of tumors, i.e., those which do not normally form tumors, to do so in mice with immune systems suppressed by sublethal irradiation, Van Pel et al., Proc. Natl. Acad. Sci. USA 76: 5282-5285 (1979); and the observation that  
35 intraperitoneally injected tum<sup>-</sup> cells of mastocytoma P815 multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst of an influx of lymphocytes and macrophages (Uyttenhove et al., J. Exp. Med. 152: 1175-1183 (1980)). Further evidence includes the observation that  
40 mice acquire an immune memory which permits them to resist subsequent challenge to the same tum<sup>-</sup> variant, even when

5 immunosuppressive amounts of radiation are administered with the following challenge of cells (Boon et al., Proc. Natl. Acad. Sci. USA 74: 272-275 (1977); Van Pel et al., supra; Uyttenhove et al., supra).

10 Later research found that when spontaneous tumors were subjected to mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these variants were able to elicit an immune protective response against the original tumor. See Van Pel et al., J. Exp. Med. 157: 1992-2001 (1983). Thus, it has been shown that it is possible to  
15 elicit presentation of a so-called "tumor rejection antigen" in a tumor which is a target for a syngeneic rejection response. Similar results have been obtained when foreign genes have been transfected into spontaneous tumors. See Fearson et al., Cancer Res. 48: 2975-1980 (1988) in this  
20 regard.

A class of antigens has been recognized which are presented on the surface of tumor cells and are recognized by cytotoxic T cells, leading to lysis. This class of antigens will be referred to as "tumor rejection antigens" or "TRAs" hereafter. TRAs may or may not elicit antibody responses. The extent to which these antigens have been studied, has been via cytolytic T cell characterization studies, in vitro i.e., the study of the identification of the antigen by a particular cytolytic T cell ("CTL" hereafter) subset. The subset  
25 proliferates upon recognition of the presented tumor rejection antigen, and the cells presenting the antigen are lysed. Characterization studies have identified CTL clones which specifically lyse cells expressing the antigens. Examples of this work may be found in Levy et al., Adv. Cancer Res. 24: 1-59 (1977); Boon et al., J. Exp. Med. 152: 1184-1193 (1980); Brunner et al., J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982); Palladino et al., Canc. Res. 47: 5074-5079 (1987). This type of analysis is  
30 required for other types of antigens recognized by CTLs, including minor histocompatibility antigens, the male specific  
35  
40

5 H-Y antigens, and the class of antigens referred to as "tum-"  
antigens, and discussed herein.

A tumor exemplary of the subject matter described supra  
is known as P815. See DePlaen et al., Proc. Natl. Acad. Sci.  
USA 85: 2274-2278 (1988); Szikora et al., EMBO J 9: 1041-1050  
10 (1990), and Sibille et al., J. Exp. Med. 172: 35-45 (1990),  
the disclosures of which are incorporated by reference. The  
P815 tumor is a mastocytoma, induced in a DBA/2 mouse with  
methylcholanthrene and cultured as both an in vitro tumor and  
a cell line. The P815 line has generated many tum<sup>-</sup> variants  
15 following mutagenesis, including variants referred to as P91A  
(DePlaen, supra), 35B (Szikora, supra), and P198 (Sibille,  
supra). In contrast to tumor rejection antigens - and this is  
a key distinction - the tum<sup>-</sup> antigens are only present after  
the tumor cells are mutagenized. Tumor rejection antigens are  
20 present on cells of a given tumor without mutagenesis. Hence,  
with reference to the literature, a cell line can be tum<sup>+</sup>,  
such as the line referred to as "P1", and can be provoked to  
produce tum<sup>-</sup> variants. Since the tum<sup>-</sup> phenotype differs from  
that of the parent cell line, one expects a difference in the  
25 DNA of tum<sup>-</sup> cell lines as compared to their tum<sup>+</sup> parental  
lines, and this difference can be exploited to locate the gene  
of interest in tum<sup>-</sup> cells. As a result, it was found that  
genes of tum<sup>-</sup> variants such as P91A, 35B and P198 differ from  
their normal alleles by point mutations in the coding regions  
30 of the gene. See Szikora and Sibille, supra, and Lurquin et  
al., Cell 58: 293-303 (1989). This has proved not to be the  
case with the TRAs of this invention. These papers also  
demonstrated that peptides derived from the tum<sup>-</sup> antigen are  
presented by the L<sup>d</sup> molecule for recognition by CTLs. P91A is  
35 presented by L<sup>d</sup>, P35 by D<sup>d</sup> and P198 by K<sup>d</sup>.

PCT application PCT/US92/04354, filed on May 22, 1992  
assigned to the same assignee as the subject application,  
teaches a family of human tumor rejection antigen precursor  
coding genes, referred to as the MAGE family. Several of  
40 these genes are also discussed in van der Bruggen et al.,  
Science 254: 1643 (1991). It is now clear that the various

5 genes of the MAGE family are expressed in tumor cells, and can  
serve as markers for the diagnosis of such tumors, as well as  
for other purposes discussed therein. See also Traversari et  
al., Immunogenetics 35: 145 (1992); van der Bruggen et al.,  
10 Science 254: 1643 (1991). The mechanism by which a protein is  
processed and presented on a cell surface has now been fairly  
well documented. A cursory review of the development of the  
field may be found in Barinaga, "Getting Some 'Backbone': How  
MHC Binds Peptides", Science 257: 880 (1992); also, see  
15 Fremont et al., Science 257: 919 (1992); Matsumura et al.,  
Science 257: 927 (1992); Latron et al., Science 257: 964  
(1992). These papers generally point to a requirement that  
the peptide which binds to an MHC/HLA molecule be nine amino  
acids long (a "nonapeptide"), and to the importance of the  
first and ninth residues of the nonapeptide.

20 Studies on the MAGE family of genes have now revealed  
that a particular nonapeptide is in fact presented on the  
surface of tumor cells, and that the presentation of the  
nonapeptide requires that the presenting molecule be HLA-A1.  
Complexes of the MAGE-1 tumor rejection antigen (the "TRA" or  
25 nonapeptide") leads to lysis of the cell presenting it by  
cytolytic T cells ("CTLs"). This observation has both  
diagnostic and therapeutic implications, as discussed herein.

Research presented in, e.g., U.S. patent application  
Serial No. 07/938,334 filed August 31, 1992 now U.S. Patent  
30 No. 5,405,940, which is the parent of the subject application  
showed that, when comparing homologous regions of various MAGE  
genes to the region of the MAGE-1 gene coding for the relevant  
nonapeptide, there is a great deal of homology. Indeed, these  
observations lead to one of the aspects of the invention  
35 disclosed and claimed therein, which is a family of  
nonapeptides all of which have the same N-terminal and C-  
terminal amino acids. These nonapeptides were described as  
being useful for various purposes which includes their use as  
immunogens, either alone or coupled to carrier peptides.  
40 Nonapeptides are of sufficient size to constitute an antigenic  
epitope, and the antibodies generated thereto were described

5 as being useful for identifying the nonapeptide, either as it exists alone, or as part of a larger polypeptide.

10 The nonapeptides were described as being useful for identifying various HLA subtypes on the surface of tumor cells, such as melanomas. Via this ability they served both as diagnostic markers and as therapeutic agents. These features are discussed infra.

15 The nucleic acid molecules which code for the nonapeptides were also described therein. These nucleic acid sequences were described as also being useful as diagnostic probes for tumor presence.

20 The application also showed how it had been found that a cellular model could be used, wherein a non-human cell can be transfected with a nucleic acid sequence coding for a human HLA molecule. The resulting transfectant could then be used to test for nonapeptide specificity of the particular HLA molecule, or as the object of a second transfection with a MAGE gene. The co-transfectant could be used to determine whether the particular MAGE based TRA is presented by the particular HLA molecule.

25 The present invention involves a binding motif or consensus sequence not previously recognized as being useful, which comprises nonapeptides which bind to HLA molecules, thereby provoking lysis by CTLs specific to complexes of the nonapeptide and the HLA molecules. The motif, or consensus sequence, may be depicted as follows:

30 (Xaa)<sub>2</sub>Asp(Xaa)<sub>2</sub>Tyr (SEQ ID NO: 23)

wherein Xaa is any amino acid. One proviso is that the motif does not cover SEQ ID NO: 12.



## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 outlines the procedure by which a 300 base pair fragment of MAGE-1 gene was identified as coding for the relevant tumor rejection antigen.

Figure 2 shows lytic studies in which cells were incubated with various MAGE 1 peptides.

Figure 3 compares lysis of mouse cells transfected with HLA-A1 genes, in the presence of the MAGE-1 nonapeptide, and when cotransfected with the sequence coding for MAGE-1.

Figure 4 compares nonapeptides from various homologous sections of MAGE genes and the nucleic acid sequences coding for these nonapeptide.

Figure 5 shows results from a chromium release assay using CTL clone 20/38 on various cell lines.

Figure 6 presents the result of assays undertaken to determine antigenic specificity of CTL clone 20/38.

Figure 7 shows the results obtained when a TNF release assay was carried out on various transfected cells.

Figure 8 sets forth the results of a lytic assay using a peptide derived from the MAGE-3 tumor rejection antigen precursor.

Figures 9A and 9B depict results of lytic assays using peptides which are examples of the motif of the invention.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

### Example 1

The 2.4 Kb BamIII fragment, described by van der Bruggen et al., Science 254: 1643 (1991), the disclosure of which is incorporated by reference, is known to contain only exons 2 and 3 of the gene coding for MAGE-1 protein. The fragment transfers expression of antigen MZ2-E to E<sup>-</sup> antigen loss cell line variant MZ2-MEL.2.2, and leads to lysis of the transfectants by E<sup>+</sup> CTLs. Previous work by DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274 (1988), and Chomez et al., Immunogenetics 35: 241 (1990), had established that small gene fragments containing antigen peptide coding sequences regularly express those antigens, even when not transfected in the form of expression vectors. In view of these

5 observations, experiments were carried out with smaller  
fragments of the 2.4 kb fragment. Various restriction enzymes  
were used to cut the 2.4 kb fragment into smaller fragments.  
The resulting, smaller fragments were cloned into plasmid  
vector pTZ18R. A 300 base pair fragment taken from exon 3 was  
10 obtained via polymerase chain reaction ("PCR") amplification,  
using oligonucleotides VDB 14:

5'-CAGGGAGCCAGTCACAAAG-3'  
(SEQ ID NO: 10)

and CHO 9:

15 5'-ACTCAGCTCCTCCCAGATTT-3'  
(SEQ ID NO: 11)

These primers amplify a 300 base pair fragment of MAGE-1,  
between positions 422 and 722 of exon 1. The fragment was  
cloned into expression vector PSVK3. The new constructs were  
20 cotransfected with plasmid pSVtkneo $\beta$  into the MZ2.MEL 2.2 cell  
lines. This was accomplished using the calcium phosphate  
precipitation method (Traversari et al., Immunogenetics 35:  
145 (1992); Wölfel et al., Immunogenetics 26: 178 (1987)),  
using 4x10<sup>6</sup> cells and 3 ug of pSVtkneo $\beta$  (Nicolas et al., CSH  
25 Conf. Cell Prolif 10: 469 (1983)), and 30 ug of the pTZ18R or  
PSVK3 constructs. The transfectants were then selected in  
medium containing neomycin analog G418. Fifteen days after  
transfection, resistant cells were tested for their ability to  
stimulate TNF production by the anti-E antigen CTL 82/30.  
30 This was accomplished by adding 100 ul samples, containing  
1500 CTL 82/30 to 4x10<sup>4</sup> transfected cells. Supernatant  
samples (50 ul) were harvested and added to 3x10<sup>4</sup> WEHI 164  
clone 13 cells (Espevik et al., J. Immunol. Meth. 95: 99  
(1986), to evaluate TNF presence. Mortality of WEHI cells was  
35 estimated 24 hours later, using an MTT colorimetric assay as  
per, e.g., Traversari et al., supra.

As shown in figure 1, these experiments identified a 300  
base pair fragment from MAGE-1 exon 3 capable of efficient  
transferring of expression of antigen MZ2-E.

#### 40 Example 2

The MAGE-1 gene belongs to a family of several highly

5 related genes. See van der Bruggen et al., supra. Prior  
experiments had noted that MAGE-2 and MAGE-3 did not direct  
expression of antigen MZ2-E. As the 300 base pair fragment  
clearly did, the homologous sections of MAGE-2 and MAGE-3  
10 genes were compared to the 300 base pair fragment.  
Differences were clear, and several 15 amino acid peptides  
were synthesized, using F-moc for transient N-terminal  
protection, in accordance with Atherton et al., J. Chem. Soc.  
1: 538 (1981). The peptides were purified by C-18 reverse  
phase HPLC, and characterized by amino acid analysis.

15 Once the peptides were secured, they were tested in lysis  
assays, using the chromium release methodology of Boon et al.,  
J. Exp. Med. 152: 1184 (1980). Briefly, 1000 <sup>51</sup>Cr labeled E<sup>-</sup>  
target cells were incubated in 96 well microplates, using  
various concentrations of peptides for 30 minutes at 37°C.

20 An equal volume of CTL containing sample was added (cell  
line 82/30), the number of CTLs being five times that of their  
target. Chromium release was measured after four hours.  
Sensitization of E<sup>-</sup> cells to lysis by the anti E CTLs was  
observed with a peptide that corresponds to codons 158-172 of  
25 the large open reading frame of MAGE-1. Shorter peptides were  
prepared and efficient lysis was observed with peptide: Glu  
Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO: 12).

The results, shown in figure 2, demonstrate that the  
first and ninth amino acids were critical for binding and  
30 effecting lysis. This is in accordance with prior reports  
stating that MHC-I molecules generally are bound by  
nonapeptides (Rotzschke et al., Nature 348: 252 (1990)).  
Figure 2 also shows that half maximum lysis was obtained at a  
peptide concentration of 5nM.

### 35 Example 3

Experiments were carried out to determine what molecule  
presented the relevant MAGE-1 antigen. To accomplish this, an  
HLA-A1 gene, as taught by Girdlestone, Nucl. Acids. Res. 18:  
6701 (1990), was transfected into a mouse cell line, P1.HTR.  
40 This line is a highly transfectable variant of mouse  
mastocytoma cell line P815. The resulting transfectants,

5 referred to as "Pl.HTR.A1", were incubated in the presence of the nonapeptide discussed supra, using the same lysis assay. Controls were also used.

Figure 3 shows that the cell line was lysed, showing that a model has been developed for screening for a lytic peptide, using a non-human cell.

10 In experiments not described herein, similar results were obtained with COS cells.

Additional experiments were also carried out, in which cell line Pl.HTR A1 was transfected with MAGE-1 cDNA. When the lytic assay of Example 2 was carried out with this co-

15 transfected cells, it was found that they were also lysed.

#### Example 4

Given the homology of the various genes within the MAGE family, a comparison was carried out to identify similarities amongst the homologous regions of the genes. These regions are shown in figure 4. These peptides and the nucleic acid sequences coding for them, are not identical, but show a great deal of homology, especially the identical first and ninth residues.

#### Example 5

25 This example, and examples 6-8 which follow, correspond to examples 37-40 of copending application Serial number 08/037,230 filed on March 26, 1993.

30 A cytolytic CTL clone "20/38" was obtained from peripheral blood lymphocytes of melanoma patient MZ2. This clone is described by Van den Eynde et al., Int. J. Cancer 44: 634-640 (1989), the disclosure of which is incorporated by reference. The CTL clone was isolated following Herin et al., Int. J. Cancer 39: 390-396 (1987), which is incorporated by reference. The assay is described herein, however.

35 Autologous melanoma cells were grown in vitro, and then resuspended at  $10^7$  cells/ml in DMEM, supplemented with 10% HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200  $\mu$ Ci/ml of Na( $^{51}$ Cr)O<sub>4</sub>. Labelled cells were washed three

40 times with DMEM, supplemented with 10 mM HEPES. These were then resuspended in DMEM supplemented with 10 mM HEPES and 10%

5 FCS, after which 100  $\mu$ l aliquots containing  $10^3$  cells, were distributed into 96 well microplates. Samples of the CTL clone were added in 100  $\mu$ l of the same medium, and assays were carried out in duplicate. Plates were centrifuged for four minutes at 100g, and incubated for four hours at 37°C in a 10 5.5% CO<sub>2</sub> atmosphere.

Plates were centrifuged again, and 100  $\mu$ l aliquots of supernatant were collected and counted. Percentage of <sup>51</sup>Cr release was calculated as follows:

15 
$$\% \text{ } ^{51}\text{Cr release} = \frac{(\text{ER}-\text{SR})}{(\text{MR}-\text{SR})} \times 100$$

where ER is observed, experimental <sup>51</sup>Cr release, SR is spontaneous release measured by incubating  $10^3$  labeled cells in 200  $\mu$ l of medium alone, and MR is maximum release, obtained by adding 100  $\mu$ l 0.3% Triton X-100 to target cells.

20 Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology.

The same method was used to test target K562 cells. When EBV-B cells were used, the only change was the replacement of DMEM medium by Hank's medium, supplemented with 25 5% FCS.

These experiments led to isolation of CTL clone 20/38.

Figure 5 presents the results of these assays. Specifically, it will be seen that the CTL clone lysed autologous melanoma cell line MZ2-MEL.3.0, but did not lyse 30 EBV-B cell lines, fibroblasts, K562 or non-autologous melanoma cell line SK-MEL-29.

#### Example 6

35 Once the CTL clone was recognized as being specific for the autologous cell line, it was tested for antigenic specificity. To do this, antigen loss variants derived from patient MZ2 were tested in the same type of chromium release assay described above. These target lines were MZ2-MEL 3.0, which is D<sup>+</sup>, E<sup>+</sup>, F<sup>+</sup>, A<sup>+</sup>, MZ2-MEL.61, which is D<sup>-</sup>, MZ2-MEL 2.2, which is E<sup>-</sup>, and MZ2-MEL.4, which is F<sup>-</sup>. In addition to CTL 40

5 clone 20/38, clones which are known to be anti-A (CTL 28/336), anti-F (CTL 76/6), and anti-E (CTL 22/13) were tested.

These results are set forth in figure 6. It will be seen that CTL clone 20/38 lysed all the cell lines leading to chromium release except D<sup>-</sup> cell line MZ2-MEL.61, thus  
10 indicating that the CTL clone is anti-D. This result was confirmed, in experiments not included herein, by experiments where TNF release by the CTL clone was observed only in the presence of melanoma lines presenting antigen D.

#### Example 7

15 Once antigen D was identified as the target molecule, studies were carried out to determine the HLA type which presented it. The experiments described in example A showed that antigen D was presented by MZ2-MEL, and this cell line's HLA specificity is known (i.e., A1, A29, B37, B44, Cw6,  
20 C.cl.10). It was also known, however, that a variant of MZ2-MEL which had lost HLA molecules A29, B44 and C.cl.10 still expressed antigen D, so these could be eliminated from consideration. Studies were not carried out on lines expressing B37, as none could be found.

25 In all, 13 allogeneic lines were tested, which expressed either HLA-A1 (10 of 13), or Cw6 (3 of 13). The cell lines were tested for their ability to stimulate release of TNF by CTL clone 20/38, using the method of Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is  
30 incorporated by reference. This assay measures TNF release via testing toxicity of supernatants on WEHI 164-13 cells.

In the assays, cell samples (3000, 10,000 or 30,000 cells) from the allogeneic lines were cultured in the presence of 1500 cells of the CTL clone, and 25 u/ml of IL-2. Twenty-  
35 four hours later, the supernatant from the culture was tested against the WEHI cells for toxicity. The results are presented in Table 1, which follows.

Eight cell lines were found to stimulate TNF release from the CTL clone 20/38. All of these lines were HLA-A1. None of  
40 the Cw6 presenting lines did so.

The cell lines were also assayed to determine MAGE

- 5 expression. All eight of the lines which stimulated TNF release expressed MAGE-3, whereas the two HLA-A1 lines which were negative did not.

Melanoma		TNF pg/ml				Expression of Mage-3	Expression of HLA-A1
		Exp 1		Exp2			
		Number of cells	-	+ CTL 20/38	-		
MZ2-MEL-61.2	50000		1		4	+++	+
MZ2-MEL-ET1	50000 1666		>120 66		>120 >120	+++	+
LY-1-MEL	30000	1	>120	1	>120	+++	+
	10000	1	>120	1	>120		
	3000	<1	114	2	>120		
MI-10221	30000	<1	>120			+++	+
	10000	<1	71				
	3000	<1	74				
LY-2-MEL	30000	1	57			+++	+
	10000	1	86				
	3000	1	91				
LY-4-MEL	30000	1	>120			+++	+
	10000	1	>120				
	3000	1	>120				
SK23-MEL	30000	1	112			++++	+
	10000	1	116				
	3000	1	105				
MI-665/2-MEL	30000	1	3	2	4	-	+
	10000	1	2	2	5		
	3000	1	5.2	1	5		
LB34-MEL	30000	1	>120			++++	+
	10000	1	>120				
	3000	1	>120				
LB45-MEL	30000	1	11	1	30	-	+
	10000	1	6	1	12		
	3000	1	2	<1	7		
NA-6-MEL	30000	1	77	5	98	+++	+
	10000	1	104	5	>120		
	3000	1	110	4	>120		
MI-13443-MEL	30000	1	>120			++++	+
	10000	1	>120				
	3000	1	>120				
LB5-MEL	30000	1	8	4	9	+	-
	10000	<1	5	4	11		
	3000	<1	5	1	5		
SK64-MEL	30000	1	4	2	5	?	-
	10000	1	2	1	5		
	3000	1	1	1	4		
LB33-MEL	30000			1	3.5	+++	-
	10000			1	4		
	3000			1	3		
LB73-MEL	50000		16			-	-

Table 1

1500 CTL 20/38 and 25 u/ml IL2 were mixed with the indicated number of cells of the different allogeneic melanomas. 24 hours later, the amount of TNF present in the supernatant was assayed by testing its cytotoxicity for WEHI-164-13 cells.



**Example 8**

In view of the results set forth in example 7, experiments were carried out to determine if antigen D was in fact a tumor rejection antigen derived from MAGE-3. To do this, recipient COS-7 cells were transfected with 100ng of the gene for HLA-A1 cloned into pCDNA I/Amp, and 100 ng of one of (a) cDNA for MAGE-1 cloned into pCDNA I/Amp, (b) cDNA for MAGE-2 cloned into pcDSR $\alpha$ , or (c) cDNA for MAGE-3 cloned into pcDSR $\alpha$ . The transfecting sequences were ligated into the plasmids in accordance with manufacturer's instructions. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 30  $\mu$ l/well of DMEM medium containing 10% Nu serum, 400  $\mu$ g/ml DEAE-dextran, 100  $\mu$ M chloroquine, and the plasmids described above. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50  $\mu$ l of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200  $\mu$ l of DMEM supplemented with 10% of FCS.

Following this change in medium, COS cells were incubated for 24 hours at 37°C. Medium was then discarded, and 1500 cells of CTL clones 20/38 were added, in 100  $\mu$ l of Iscove's medium containing 10% pooled human serum, supplemented with 25 u/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. These results are shown in Figure 7. It will be seen that the CTL clone was strongly stimulated by COS7 cells transfected with HLA-A1 and MAGE-3, but not by the cells transfected with the other mage genes. This leads to the conclusion that antigen D is a tumor rejection antigen derived from the tumor rejection antigen precursor coded by gene MAGE-3, and that this TRA is presented by HLA-A1 molecules.

5        **Example 9**

          Further experiments were carried out using peptide  
          Glu Val Asp Pro Ile Gly His Leu Tyr (SEQ ID NO: 17)  
which is derived from the gene "MAGE-3".

10        The peptide was prepared in the same manner as were the  
          peptides of example 2. The chromium release assay described  
          in that example was also used. Cell line MZ2-MEL 61.2, which  
          is an antigen D loss variant of MZ2.MEL43 was labelled with  
          <sup>51</sup>Cr, and was then tested with antigen D specific cytolytic  
15        cell clone CTL 20/38, and varying concentrations of the  
          peptide. MZ2-MEL61.2 and CTL 20/38 were combined in a 1.5:1  
          ratio, together with the peptide at varying concentrations.  
          The mixture was incubated for four hours, after which chromium  
          release was measured. As a control, chromium labelled MZ2-  
          MEL.43 was used.

20        The results, presented in figure 8, show that the peptide  
          does act as a tumor rejection antigen in that the cytolytic T  
          cell clones recognize and lyse the targeted cells.

**Example 10**

25        This example proves that the first amino acid of the  
          nonamer is not essential for the binding of peptides to the  
          HLA-A1 molecule, nor to recognition and lysis by CTLs. The  
          key peptide in this experiment is

30        Ala Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO: 14)  
          which differs from the peptide of SEQ ID NO: 12 only in that  
          position 1 (the "N terminal" amino acid), is Ala, rather than  
          Glu.

35        The cell line BM21 is well known, and is HLA-A1 positive.  
          Samples of the cell line were chromium labeled by incubating  
          10<sup>6</sup> cells with <sup>51</sup>Cr, for one hour at 37°C. The incubation took  
          place in the presence of anti-HLA Class I monoclonal antibody  
          W6/32, which stabilized the HLA molecules, in order to  
          facilitate the experiments which followed. Then, a  
          competitive binding experiment was set up, wherein varying  
40        concentrations of competitor peptide (0.03 to 100 µM) were  
          mixed with the cells at 1000 cells/well. Fifteen minutes  
          later, the peptide of SEQ ID NO: 17 (i.e., Glu Val Asp Pro Ile

5 Gly His Leu Tyr) was added, at a concentration of 0.25  $\mu$ M. Fifteen minutes following this addition, cells of CTL clone 20/38, known to recognize and lyse complexes of SEQ ID NO: 17 and HLA-A1 molecules, were added at a lymphocyte to target ratio of 10:1 and incubated at 37°C. Lysis was measured four  
10 hours later, using the  $^{51}\text{Cr}$  methodology described herein. The results are presented in figures 9A and 9B.

In figure 9A, peptides based upon the deduced amino acid sequence of MAGE-1 were tested, whereas in figure 9B, homologous peptides from various MAGE peptides were used. Figure 9A, in particular, shows that a peptide without Glu in  
15 first position was effective in competing with a peptide known to bind to an HLA molecule. Specifically, what was tested was the ability of a series of "Ala" substituted peptides to prevent MAGE-3 derived peptide MZ-2D from binding to HLA-A1  
20 molecules. This was tested by observing decrease in lysis by CTLs specific for complexes of HLA-A1 and MZ-2D. As noted, in the figures, there was competition, indicated by a decrease in lysis.

It is well known that cells of a particular CTL recognize and lyse specific complexes of peptide and HLA only. The fact  
25 that CTLs specific to MZ-2D and HLA-A1 do not lyse complexes of competing peptide and HLA-A1, does not mean these complexes would not provoke lysis. Rather, such complexes are expected to be sufficient to provoke proliferation of additional CTLs,  
30 specific to complexes of an HLA molecule and a competitor nonapeptide.

The foregoing examples show that nonapeptides of formula (Xaa)<sub>2</sub>Asp(Xaa)<sub>2</sub>Tyr (SEQ ID NO: 123 are presented by HLA molecules, and cells presenting the complex of HLA and a  
35 nonapeptide in accordance with this can be lysed by specific CTL cells generated in response to such complexes. This observation indicates that the nonapeptides of the invention may be used both therapeutically and diagnostically.

In the case of the latter category of use, the  
40 nonapeptide may be used, for example, to identify tumors expressing a particular HLA molecule, or cancer cells per se.

5 One contacts a cancer cell containing sample or a tumor cell  
with the nonapeptide which binds thereto, and combines the  
material with a CTL sample specific for the complex. If lysis  
10 ensues, then the tumor/cancer cell can be identified as a  
particular HLA presenter. More particular analysis can show  
that the cancer cells are, e.g., HLA-A1 presenters, or  
presenters of other HLA types, such as other HLA-A types.

Therapeutically, there are two major ways in which the  
nonapeptide may be used. In an in vivo therapeutic approach,  
15 the nonapeptide may be administered in a way which targets it  
to tumors to be treated. This can be done via direct  
injection, time release administration, coupling to tumor  
specific antibodies, and so forth. Upon binding to HLA-A  
molecules, there is a CTL response, leading to lysis of the  
20 tumor. Of course, in such a therapeutic approach, the  
nonapeptide is administered in an amount sufficient to lead to  
lysis of the tumor. This amount will vary, based upon the  
particular patient, the type and size of the tumor, and so  
forth.

A further aspect of the invention is the use of these  
25 peptides, either alone or in composition form, to provoke or  
to stimulate a response by CTLs which are silent, or otherwise  
inactive. In composition form, these peptides are combined  
with any of the well known adjuvants useful in stimulating an  
immune response. Upon administration, the silent or inactive  
30 CTLs proliferate and target any tumor cells presenting  
complexes of peptides and HLA molecules on their surface.

An "in vitro" form of therapy is also contemplated. As  
indicated supra, when HLA-A molecules bind to the nonapeptide,  
if contacted with the CTLs specific for the peptide/HLA  
35 complex, a CTL proliferative response occurs. As the CTLs are  
the agents of tumor lysis in vivo, the resulting expanded  
populations may be administered to the patient. The CTLs can  
be expanded by using the patient's own blood or any other  
source of CTLs, or by contact to samples of peptide specific  
40 CTLs which have previously been established. In this regard,  
note that CTL 20/38, discussed supra had been available for

5 some time as was the methodology for its development.

Therapies of the type described herein are particularly useful for melanoma. For example, analysis of samples has shown that about 26% of the caucasian population at large presents HLA-A1 allele. Thus, at the least, 26% of the caucasian melanoma population may be considered as potential subjects for therapy with the peptide. The patients may also be treated with proliferative cells which have complexes of HLA-A1 and the nonapeptide presented on their surface.

10 The nucleic acid sequences, as indicated, may be used in a variety of ways. MAGE genes are expressed in tumors, and thus the nucleic acid sequences may be used as probes to identify tumor cells. This can be accomplished via labelled hybridization probes, PCR, or any of the various nucleic acid probe based assays known to the art.

15 The development of the non-human cell lines described herein presents a unique way to carry out some of the features of the invention described herein. The examples show, e.g., that the CTLs recognize the complex of HLA and nonapeptide, and do not appear to differentiate between the cell types which present the complexes. Thus, the isolated, non-human cell lines of the invention can be used to generate CTLs, and to identify their presence in human samples.

20 As indicated, the invention also involves isolated non-human cell lines transfected with both an HLA-A1 gene, and a sequence coding for the nonapeptide. One is not limited to transfection with one HLA coding gene and one MAGE peptide, and indeed the invention contemplates polytransfected cells, which may contain more than one HLA gene and more than one MAGE antigen coding sequence. Given the finding that both nonapeptides of the disclosed motif are presented by a common HLA molecule supports this contention. Such cells may be regarded as universal effector cells, as the presence of appropriate pairs of HLA and peptide on the surface will lead either to identification of specific CTLs of choice, or to generation of CTL proliferation in a therapeutic context. Such cells, be they cotransfected or polytransfected, may

5       serve as vaccines when combined with a suitable adjuvant, such  
as those well known to the art. Treatment of various  
cancerous conditions, such as melanoma and breast cancer, may  
be carried out using these transfectants.

10       The terms and expressions which have been employed are  
used as terms of description and not of limitation, and there  
is no intention in the use of such terms and expressions of  
excluding any equivalents of the features shown and described  
or portions thereof, it being recognized that various  
modifications are possible within the scope of the invention.

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22

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- (2) INFORMATION FOR SEQ ID NO: 1:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-1 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAAGCAGACC CCACCGGCCA CTCCTAT

27

- (2) INFORMATION FOR SEQ ID NO: 2:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-2 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAGTGGTCC CCATCAGCCA CTTGTAC

27

- (2) INFORMATION FOR SEQ ID NO: 3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-21 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAAGTGGTCC GCATCGGCCA CTTGTAG

27



23

- (2) INFORMATION FOR SEQ ID NO: 4:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-3 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAAGTGGACC CCATCGGCCA CTTGTAC

27

- (2) INFORMATION FOR SEQ ID NO: 5:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-4 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAAGTGGACC CCGCCAGCAA CACCTAC

27

- (2) INFORMATION FOR SEQ ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: genomic DNA
  - (ix) FEATURE:
    - (A) NAME/KEY: MAGE-41 nonapeptide coding sequence
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAAGTGGACC CCACCAGCAA CACCTAC

27

24

- (2) INFORMATION FOR SEQ ID NO: 7:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 27 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: MAGE-5 nonapeptide coding sequence  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAAGCGGACC CCACCAGCAA CAACTAC

27

- (2) INFORMATION FOR SEQ ID NO: 8:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 27 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: MAGE-51 nonapeptide coding sequence  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAAGCGGACC CCACCAGCAA CACCTAC

27

- (2) INFORMATION FOR SEQ ID NO: 9:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 27 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: MAGE-6 nonapeptide coding sequence  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAAGTGGACC CCATCGGCCA CGTGTAC

27

25

- (2) INFORMATION FOR SEQ ID NO: 10:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 19 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAGGGAGCCA GTCACAAAG

19

- (2) INFORMATION FOR SEQ ID NO: 11:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ACTCAGCTCC TCCCAGATTT

20

- (2) INFORMATION FOR SEQ ID NO: 12:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Glu Ala Asp Pro Thr Gly His Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 13:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 8 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Glu Ala Asp Pro Thr Gly His Ser  
5

26

- (2) INFORMATION FOR SEQ ID NO: 14:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 8 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Ala Asp Pro Thr Gly His Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 15:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Glu Val Val Pro Ile Ser His Leu Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 16:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Glu Val Val Arg Ile Gly His Leu Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 17:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Glu Val Asp Pro Ile Gly His Leu Tyr  
5

27

- (2) INFORMATION FOR SEQ ID NO: 18:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Glu Val Asp Pro Ala Ser Asn Thr Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 19:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Glu Val Asp Pro Thr Ser Asn Thr Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 20:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Glu Ala Asp Pro Thr Ser Asn Thr Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 21:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Glu Ala Asp Pro Thr Ser Asn Thr Tyr  
5

28

- (2) INFORMATION FOR SEQ ID NO: 22:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Glu Val Asp Pro Ile Gly His Val Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 23:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 8 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Xaa Asp Xaa Xaa Xaa Xaa Xaa Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 24:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Ala Ala Asp Pro Thr Gly His Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 25:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Glu Ala Asp Ala Thr Gly His Ser Tyr  
5

29

- (2) INFORMATION FOR SEQ ID NO: 26:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Glu Ala Asp Pro Ala Gly His Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 27:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Glu Ala Asp Pro Thr Ala His Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 28:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Glu Ala Asp Pro Thr Gly Ala Ser Tyr  
5

- (2) INFORMATION FOR SEQ ID NO: 29:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Glu Ala Asp Pro Thr Gly His Ala Tyr  
5

**We claim:**

1. Isolated nonapeptide of formula

(Xaa)<sub>2</sub>Asp(Xaa)<sub>2</sub>Tyr (SEQ ID NO: 23)

wherein said nonapeptide binds to an HLA molecule on a cell and provokes lysis by cytolytic T cells specific for a complex of said HLA molecule and said nonapeptide, with the proviso that said nonapeptide is not SEQ ID NO: 12.

2. The isolated nonapeptide of claim 1, wherein the first amino acid is Glu or Ala.

3. The isolated nonapeptide of claim 1, wherein the second amino acid is Ala or Val.

4. The isolated nonapeptide of claim 1, wherein the fourth amino acid is Pro, Ala or Arg.

5. The isolated nonapeptide of claim 1, wherein the fifth amino acid is Thr, Ile or Ala.

6. The isolated nonapeptide of claim 1, wherein the sixth amino acid is Gly, Ala or Ser.

7. The isolated nonapeptide of claim 1, wherein the seventh amino acid is His, Ala or Asn.

8. The isolated nonapeptide of claim 1, wherein the eighth amino acid is Ser, Leu, Thr, Ala or Val.

9. The isolated nonapeptide of claim 1, selected from the group consisting of

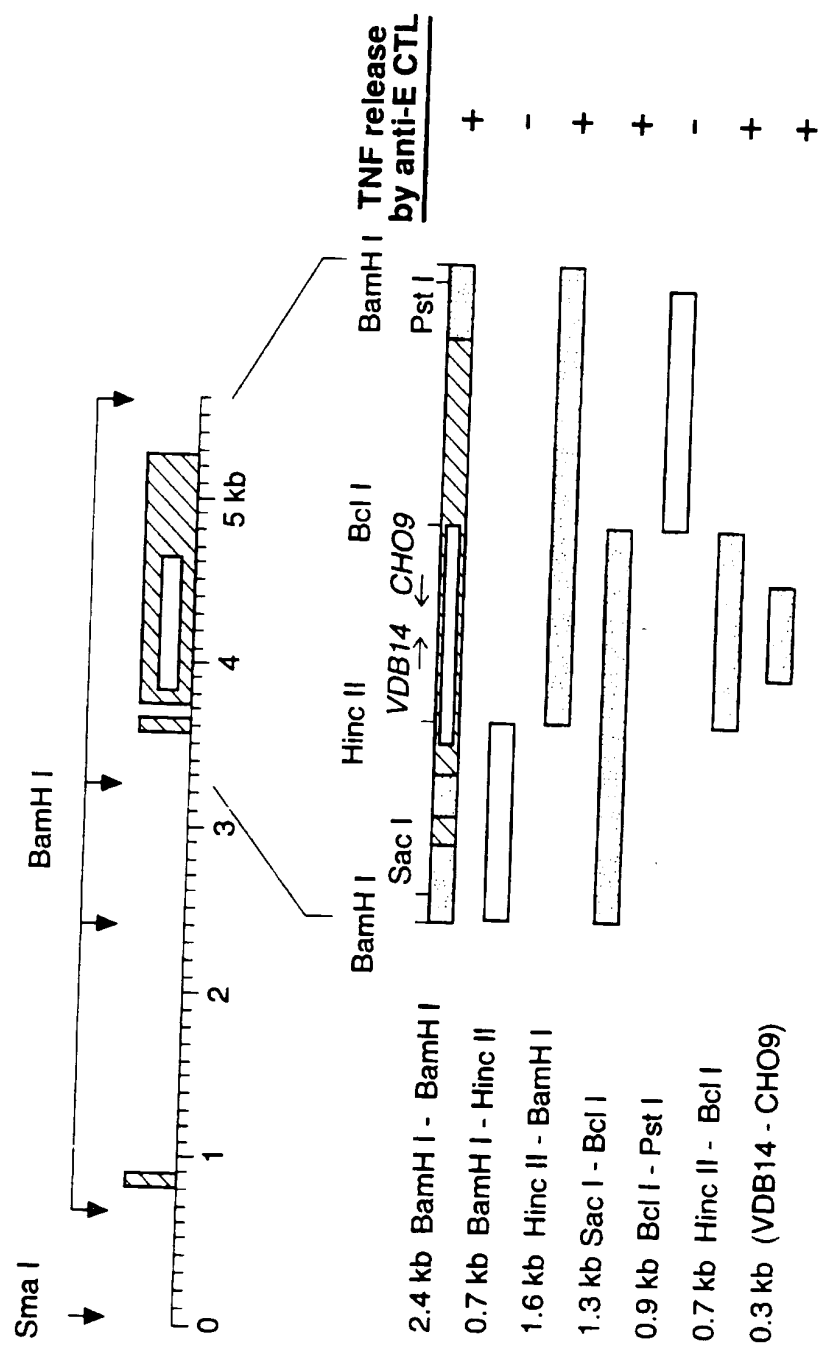
Ala Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO: 24),  
Glu Ala Asp Ala Thr Gly His Ser Tyr (SEQ ID NO: 25),  
Glu Ala Asp Pro Ala Gly His Ser Tyr (SEQ ID NO: 26),  
Glu Ala Asp Pro Thr Ala His Ser Tyr (SEQ ID NO: 27),  
Glu Ala Asp Pro Thr Gly Ala Ser Tyr (SEQ ID NO: 28), and  
Glu Ala Asp Pro Thr Gly His Ala Tyr (SEQ ID NO: 29).

10. The isolated nonapeptide of claim 1, consisting of amino acid sequence

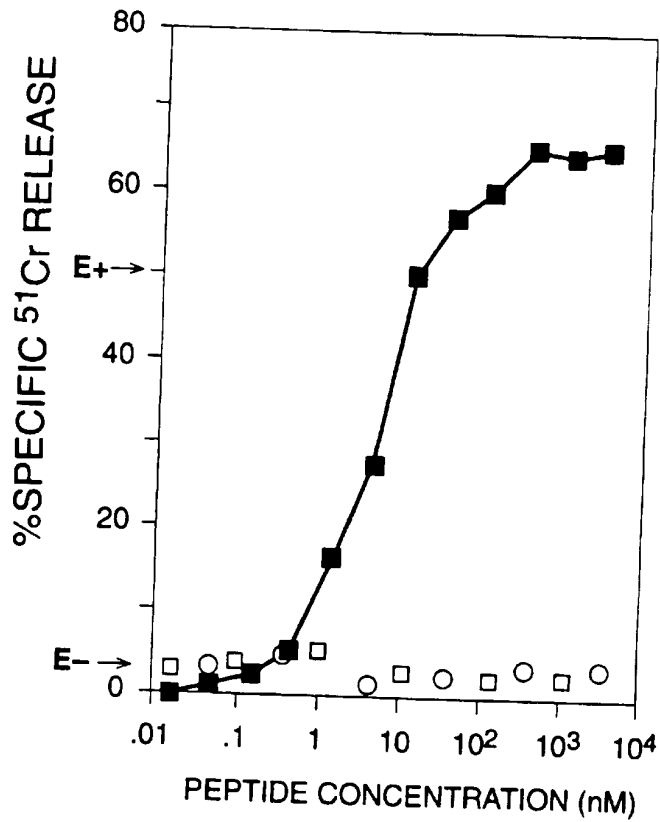
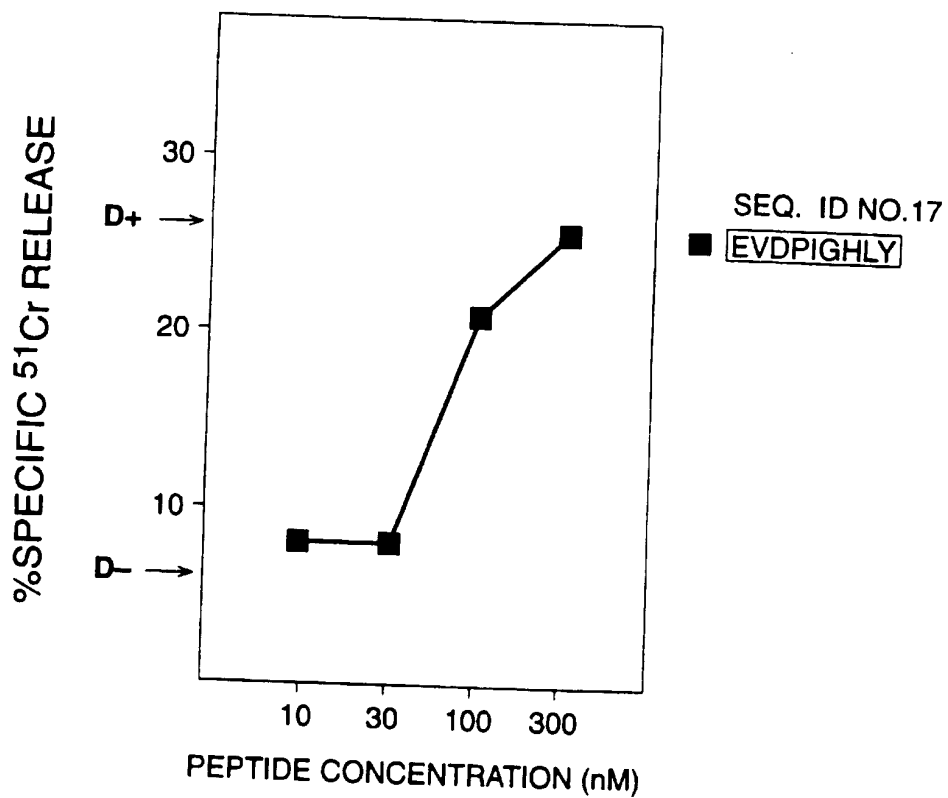
Ala Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO: 24).



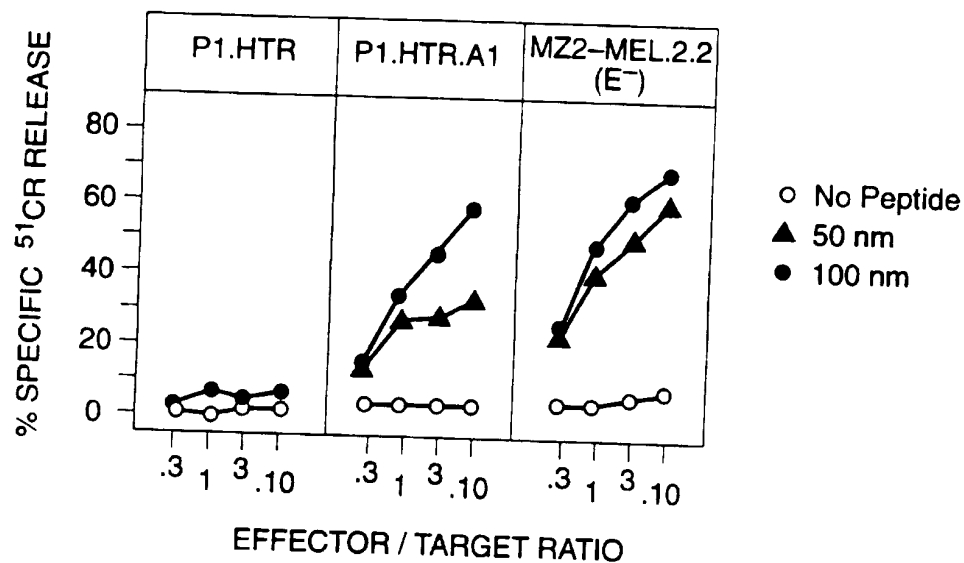
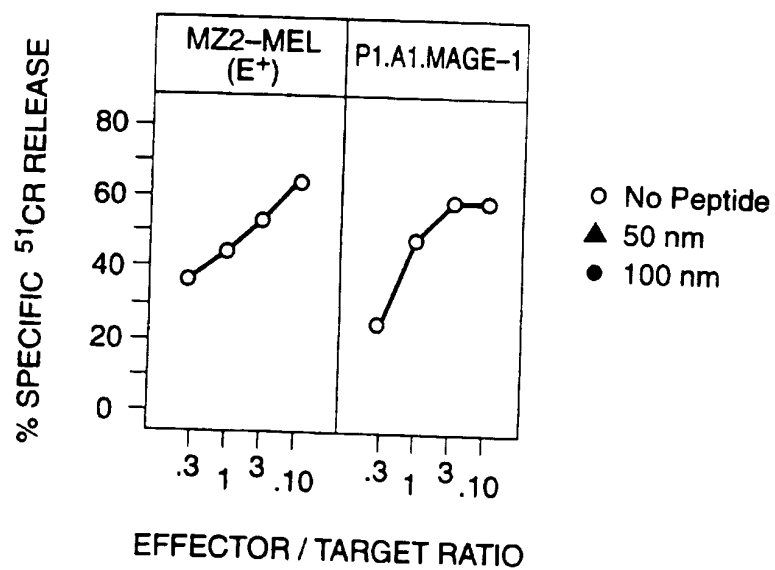
FIG. 1



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**FIG. 8**

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**FIG. 3A****FIG. 3B**

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## FIG. 4

MAGE 1	E	A	D	P	T	G	H	S	Y
MAGE 2	E	V	V	P	I	S	H	L	Y
MAGE 21	E	V	V	R	I	G	H	L	Y
MAGE 3	E	V	D	P	I	G	H	L	Y
MAGE 4	E	V	D	P	A	S	N	T	Y
MAGE 41	E	V	D	P	T	S	N	T	Y
MAGE 5	E	A	D	P	T	S	N	T	Y
MAGE 51	E	A	D	P	T	S	N	T	Y
MAGE 6	E	V	D	P	I	G	H	V	Y

MAGE 1	GAA	GCA	GAC	CCC	ACC	GGC	CAC	TCC	TAT
MAGE 2	GAA	GTG	GTC	CCC	ATC	AGC	CAC	TTG	TAC
MAGE 21	GAA	GTG	GTC	CGC	ATC	GGC	CAC	TTG	TAC
MAGE 3	GAA	GTG	GAC	CCC	ATC	GGC	CAC	TTG	TAC
MAGE 4	GAA	GTG	GAC	CCC	GCC	AGC	AAC	ACC	TAC
MAGE 41	GAA	GTG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 5	GAA	GCG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 51	GAA	GCG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 6	GAA	GTG	GAC	CCC	ATC	GGC	CAC	GTG	TAC

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FIG. 5A

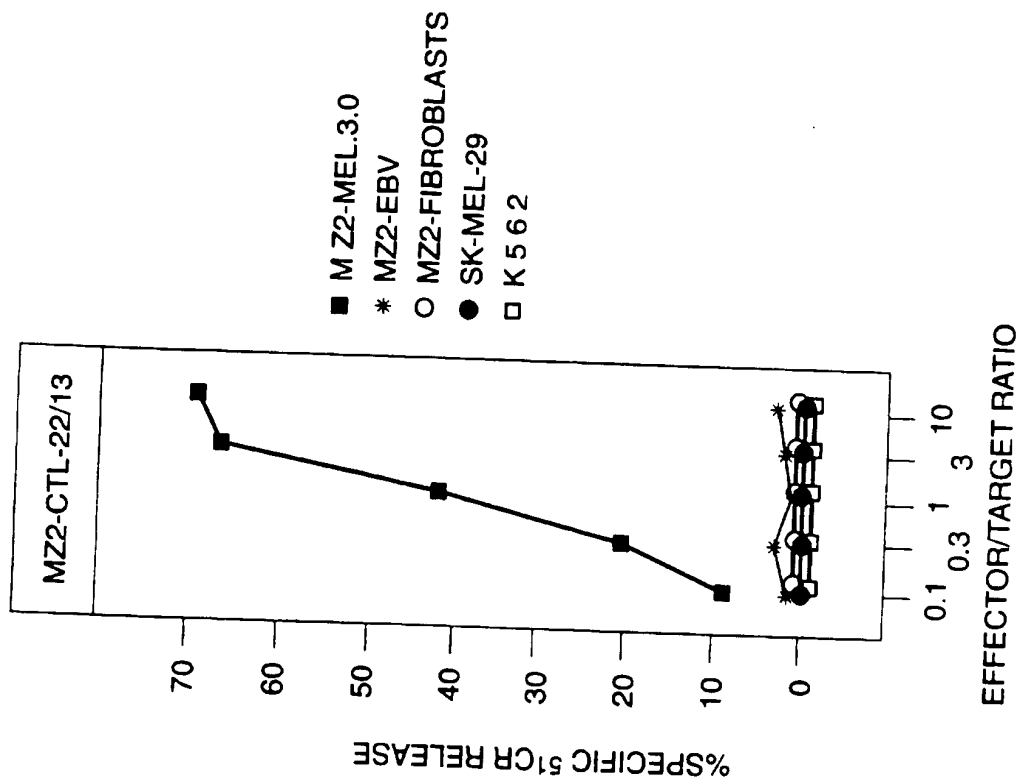
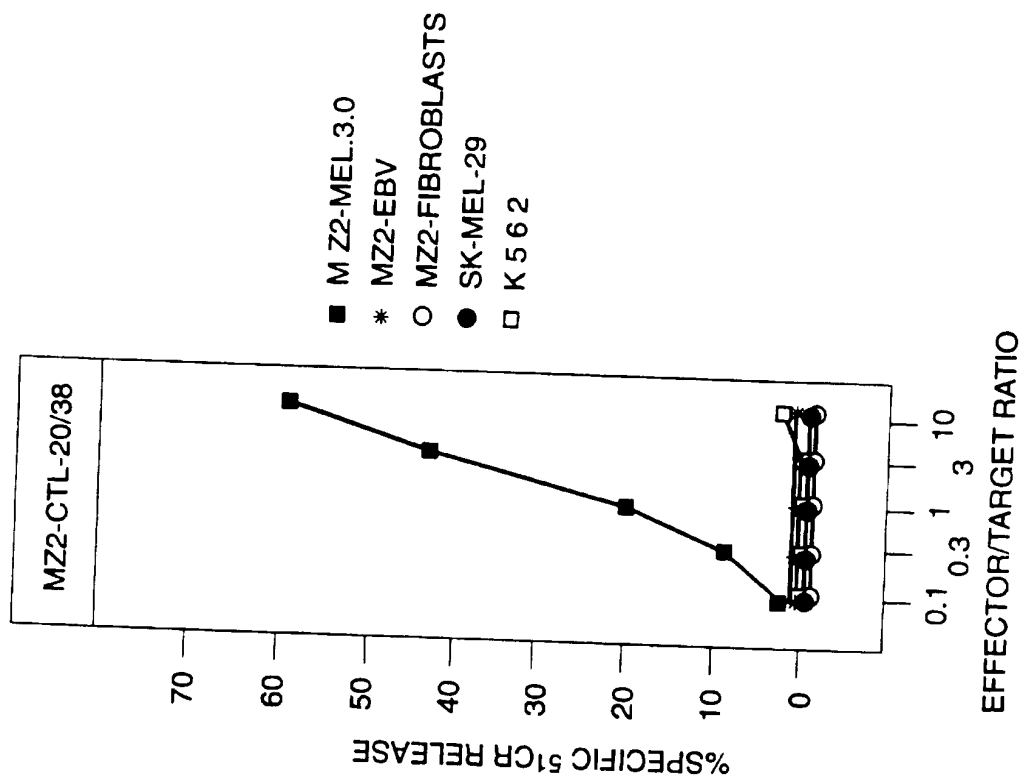
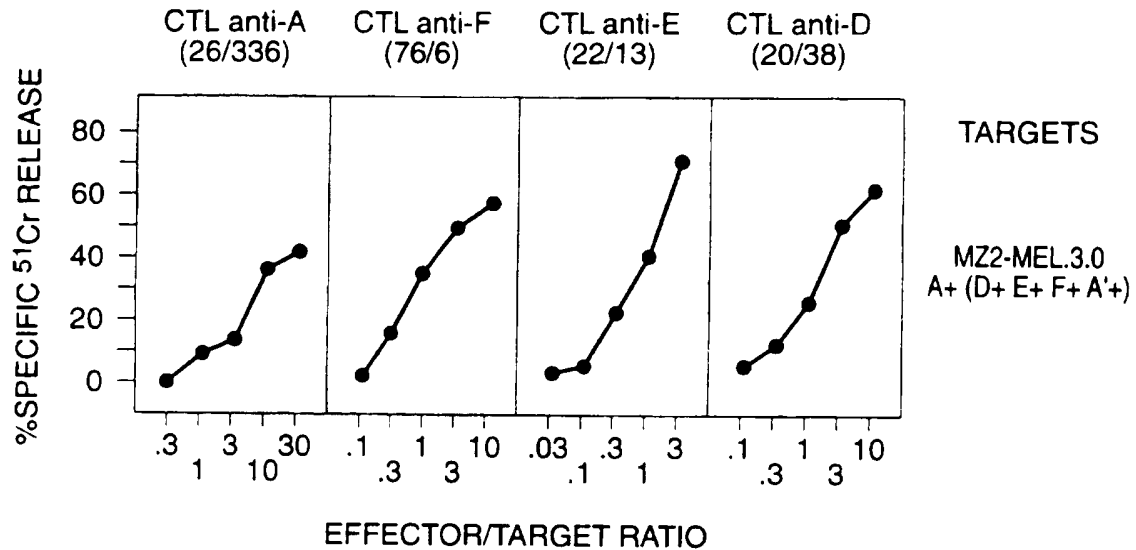
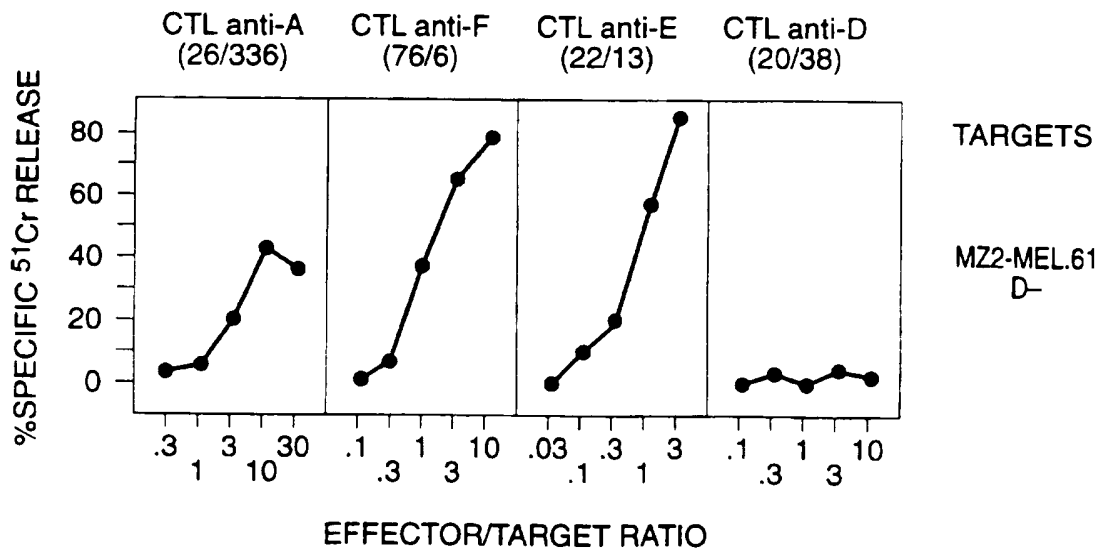


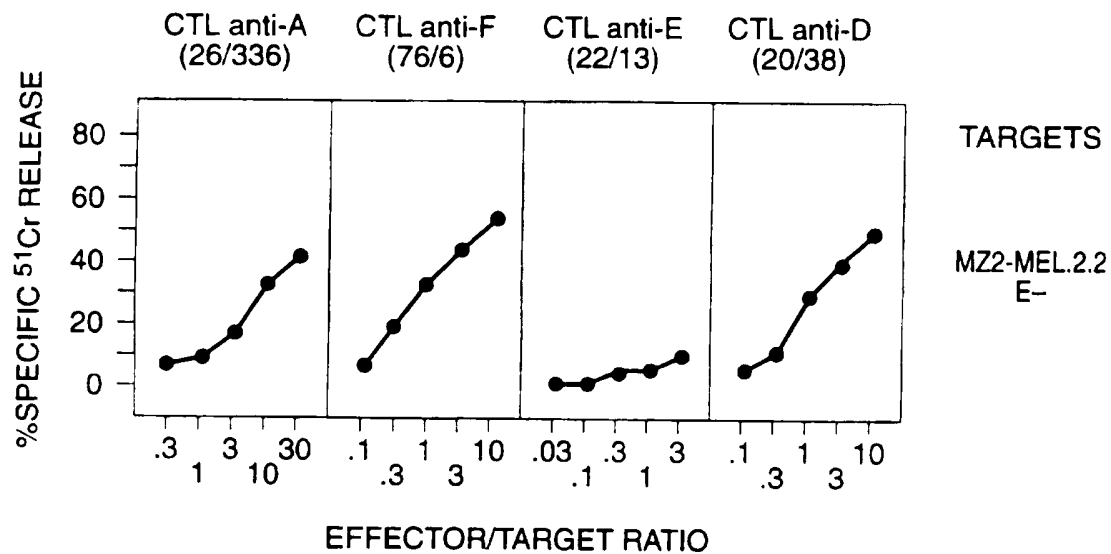
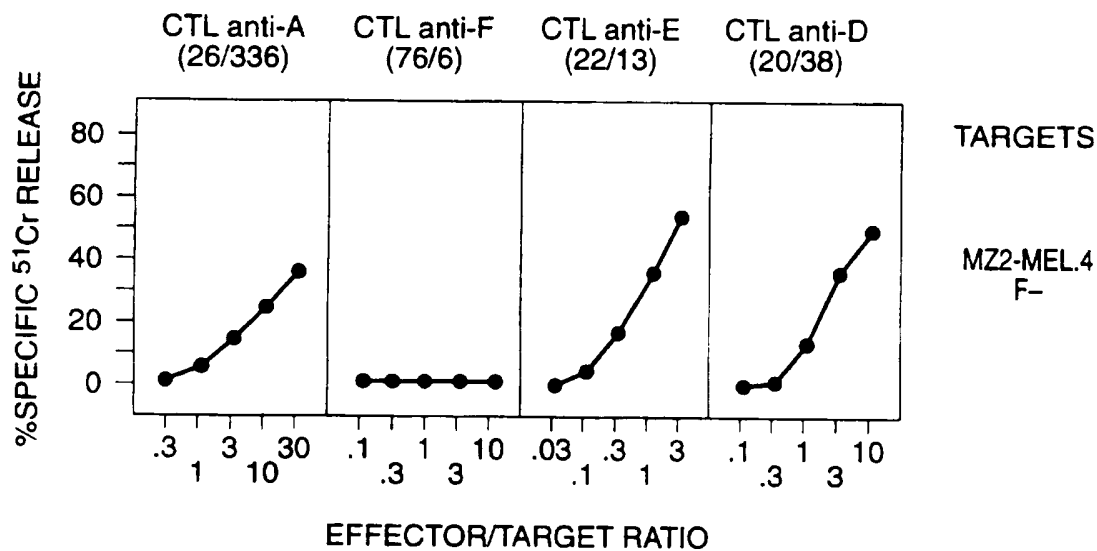
FIG. 5B



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**FIG. 6A****FIG. 6B**

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**FIG. 6C****FIG. 6D**

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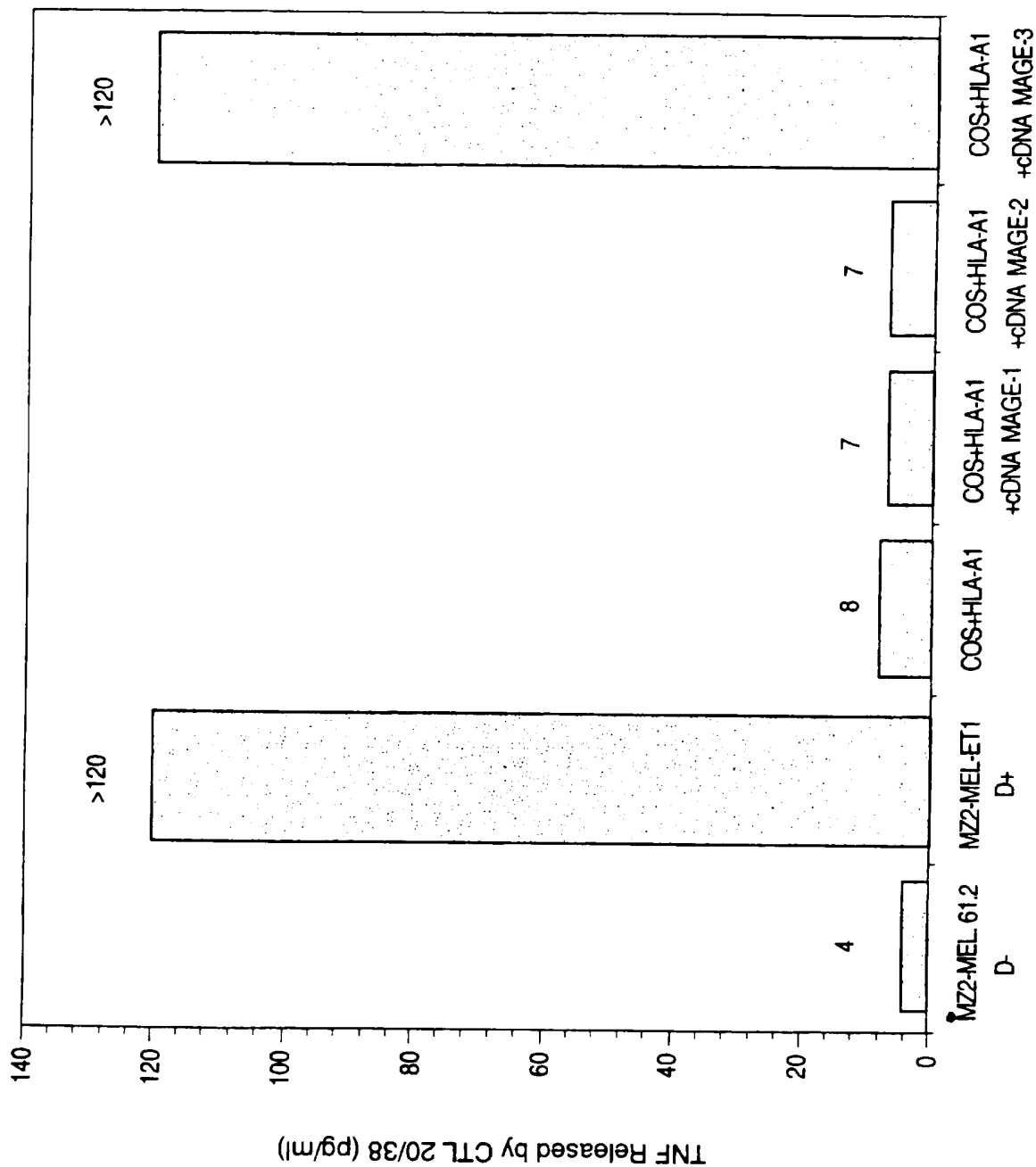


FIG. 7



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FIG. 9A

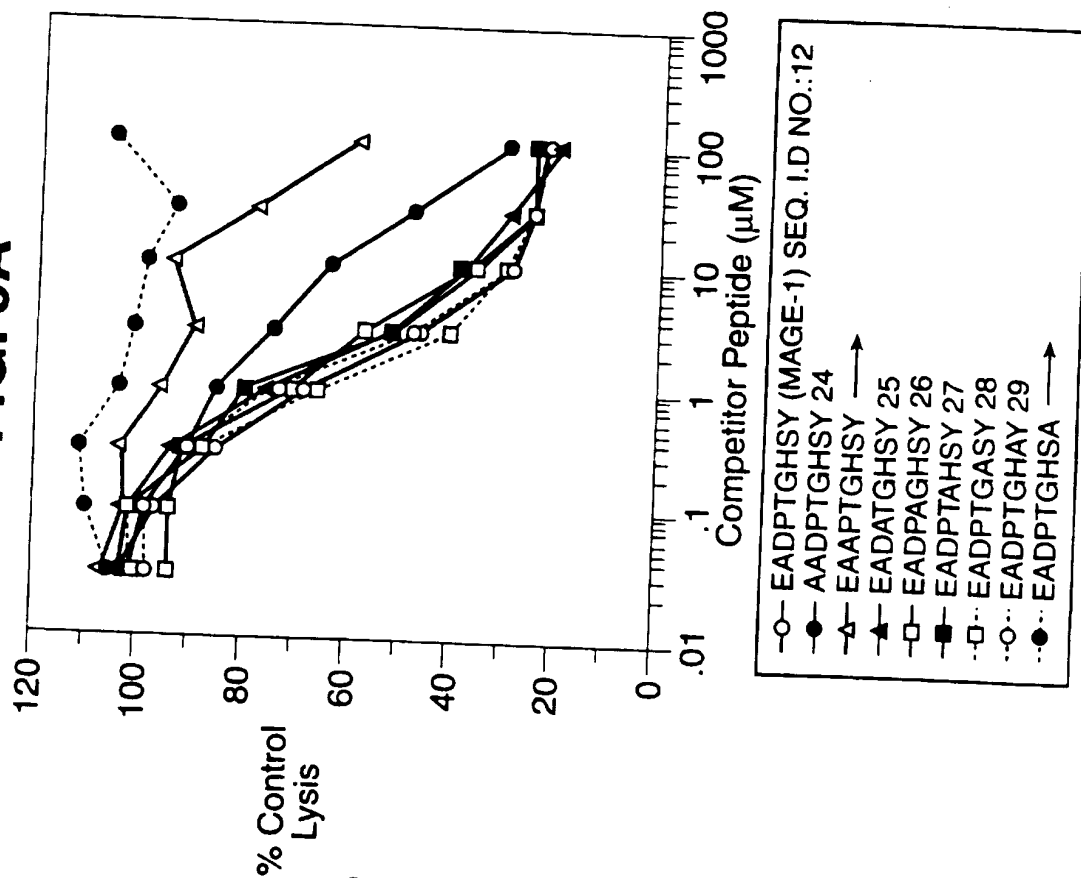
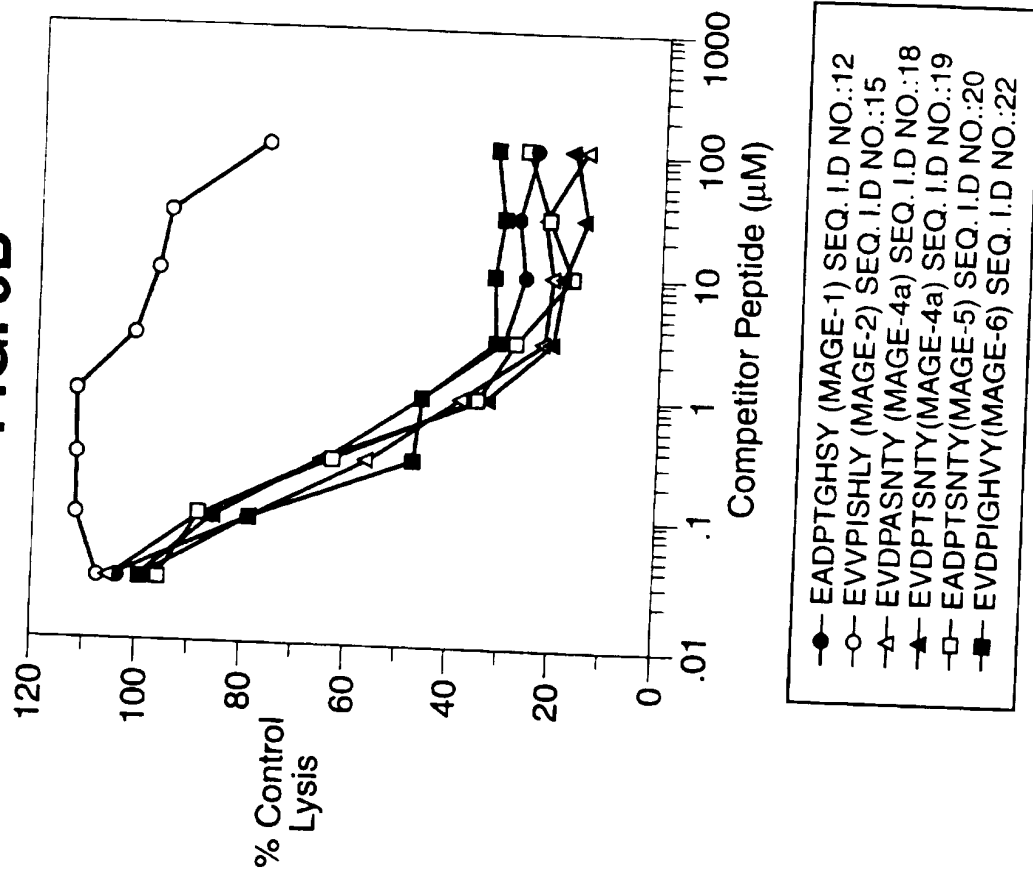


FIG. 9B



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/01489

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07K 7/04; A61K 38/00

US CL : 530/328; 424/185.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328; 424/185.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Examiner's MAGE peptide files.Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, DIALOG (file BIOCHEM). Key words: MAGE, tumor rejection antigen, peptide and CTL**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US, A, 5,405,940 (BOON et al.) 11 April 1995, see claims.	1-10
A	BIOCHEMICAL BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 202, No. 1, published 15 July 1994, Ding et al., "Cloning and Analysis of MAGE-1-Related Genes". Pages 549-555, see entire document.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance		
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search

11 APRIL 1996

Date of mailing of the international search report

24 APR 1996

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